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ARTICLE

Contents of Vitamin C, Carotenoids, Tocopherols, and Tocotrienols in the Subtropical Plant Species *Cyphostemma digitatum* as Affected by Processing

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The subtropical plant species *Cyphostemma digitatum*, Vitaceae, is used in central Yemen in traditional medicine, as a culinary herb, and as a source of food flavoring. The contents of vitamin C, vitamin E, and carotenoids and changes caused by common processing were investigated. Carotenoids were determined by reversed phase C30-high-performance liquid chromatography (HPLC) with diode array detection at 470 nm, while tocopherols and tocotrienols were analyzed by using normal phase HPLC with fluorescence detection (excitation, 292 nm; emission, 330 nm). Ascorbic acid was determined spectrophotometrically after reaction with DNP by measuring the absorbance at 520 nm. For the raw material and for the processed commercial food product, both in dried form, reasonable quantities of carotenoids were found in the raw material as follows: lutein, 18.89 ± 0.73 mg/100 g; zeaxanthin, 9.46 ± 0.30 mg/100 g; canthaxanthin, 0.21 ± 0.01 mg/100 g; β-cryptoxanthin, 0.67 ± 0.03 mg/100 g; and β-carotene, 14.60 ± 0.46 mg/100 g. Household processing reduced the carotenoid contents dramatically; only β-carotene sustained the processing. Likewise, vitamin C, 49.50 ± 0.01 mg/100 g in the raw material and 20.30 ± 0.02 mg/100 g in the processed material, was affected negatively by processing; only 41% was retained after processing. In contrast, the outstanding high content of vitamin E, 82.74 ± 0.63 mg/100 g in the raw material, was increased by processing to 101.20 ± 1.38 mg/100 g; it was found in different forms, some of which were rare in other sources.

KEYWORDS: *Cyphostemma digitatum*; antioxidant activity; carotenoids; provitamin A; vitamin C; vitamin E; processing effect; Yemen

INTRODUCTION

Cyphostemma digitatum (Vitaceae) is a perennial, climbing, succulent undershrub with compound fleshy leaves and tendrils. The leaves are petiolate, digitately 3−5 foliolate; leaflets are ovate and dentate. It flowers in pedunculate axillary cymes, and the fruits are one-seeded, red fleshy berries (**Figure 1**). *C. digitatum* usually occurs between 1400 and 2500 m a.s.l., often on cliffs and with preference for shaded stony places such as gullies and terraces walls. It is usually associated with *Acacia* spp., *Agava* spp., *Senecio hadiensis*, *Clematis* spp., and *Euphorbia* spp. (*1*).

The species is strongly declining in its natural habitats, because it becomes a commercial food product after processing. Therefore, it has disappeared completely from many regions in the southwestern highlands of Yemen because of intensive gathering. The leaves and fleshy young stem branches are used in dried form after processing. People started to cultivate it by removing the plant completely from its original sites and replanting it in gardens. The plant is used mainly as a food flavoring, but it is also a main constituent of traditional Yemeni soup (Marak). Besides that, it has been described to be used as a medication for gastroenteritis, fatigue, vomiting, and headache, against malaria, and for general health support.

In general, carotenoids are lipid-soluble pigments found in many vegetable crops that are reported to have health benefits against cancer and eye diseases when consumed in the diet (*2*). Structurally, carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). As carotenoids are responsible for the colors of many plants, fruits, and flowers, more than 700 carotenoids have been isolated from natural sources. They serve as light-harvesting complexes (with proteins) in photosynthesis (*3*). Functionally, some carotenoids are important in human nutrition as a source of vitamin A (e.g., β-carotene) and as a prevention agent for cancer and heart disease (e.g., lycopene) (*3*). A high intake of β-carotene also might decrease the risk of cancer in humans (*4*). In addition, carotenoids are the precursors of many important chemicals responsible for the flavor of foods and fragrance of flowers (*3*). Vitamin C, being a water-soluble antioxidant, may reductively regenerate oxidized vitamin E (*5*).

Vitamin E became important clinically to be determined; age-dependent reference intervals were constructed; in a cross-sectional survey, the influence of age, sex, and season of sampling on vitamin E plasma concentrations in 208 Swiss individuals aged

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Figure 1. *C. digitatum* mature plant before harvesting, showing the fleshy leaves and different flowering and fruiting stages.

from 0.4 years to 38.7 years was studied (*6*). Age was a significant predictor of plasma vitamin E concentrations; no sex-related differences were observed. The season of sampling affected α-tocopherol, γ-tocopherol, and cholesterol concentrations; they were higher in winter and spring than in the other seasons. The ratios of plasma α-tocopherol to cholesterol were not affected by age (*6*).

Vitamin C, vitamin E, and β-carotene all displayed antioxidant activity and thus provided a cellular defense against reactive oxygen species, which could damage the DNA (*7*). Serum β-carotene and vitamin E levels showed a strong protective association with lung cancer and suggestive protective associations with melanoma and bladder cancer (*8*). Additionally, cooperative interactions between vitamin E and vitamin C in protecting against lipid peroxidation in liposomes have been examined (*9*). Barclay et al. (*9*) briefly discussed the mechanism of this synergism between vitamin E and vitamin C. Simultaneous determination of vitamins C and E and carotenoids became a target for many clinical screening studies in different samples associated with many illnesses (*10*).

In a duration-dependent manner, vitamin E and β-carotene provide protection against erythema in humans and may also be useful for diminishing sensitivity to ultraviolet light. The supplementation with carotenoids or a combination of carotenoids and vitamin E for 12 weeks at dosages exceeding dietary intakes of these antioxidants increased the basal protection of skin against erythema (*11*). Nowadays, the effects of β-carotene and vitamins C and E sets of supplementation are a hot topic of research (*12*). Consequently, the simultaneous determination of this set of functional food ingredients was done for many food products (*5*, *13*). The chloroplasts contain carotenoids (e.g., β-carotene), vitamin E (tocopherols), and vitamin C (L-ascorbic acid), which cooperatively act as antioxidants. This led to a study of biomass production by the aerial microalga *Trentepohlia aurea* in liquid culture for the simultaneous production of β-carotene, vitamin E, and vitamin C under various conditions (*5*).

Multichannel, flash kinetic spectroscopy with microsecond time resolution has been used for investigation of the photochemical and photophysical behavior of vitamin E forms and its interaction with carotenoids (*14*). Although β-carotene, vitamin E, and vitamin C are antioxidants, it has been suggested that they may also serve as pro-oxidants in certain situations. They may promote oxidation via their action upon certain molecules, such as cupric (Cu^{2+}) and ferric (Fe^{3+}) ions, by the failure to regenerate after serving in their role as antioxidants (*12*).

It is well-known that vitamin A deficiency (VAD), xerophthalmia, and age-related macular degeneration (AMD) are primarily due to an inadequacy of provitamin A and macular pigments in the diet. This is the reason why VAD and AMD are well-known as serious public health problems among children and adults in Yemen and many developing countries (*15*). Consequently, much research has been concentrated on the identification of carotenoids and provitamin A in common and in less familiar green leafy vegetables (GLVs) and on its way of processing in different cultures to illustrate its valuable importance for human health and nutrition among them. It is known that vitamin C, vitamin E, and β-carotene were more decreased the higher the temperature and pressure were in the processing of hot and sweet pepper (*13*). The bioavailability of carotenoids seems to be affected by postharvest and processing activities; relevant studies emphasized the importance of carotenoids enhancement in vegetable crops and the need to characterize potential changes in carotenoids composition during cultivation, storage, and processing before consumer purchase (*2*).

The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for these potent antioxidant and anticancer activities, and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (*16*). In our previous paper, the remarkably high antioxidant capacity of *C. digitatum* was quantified by four different methods in the context of standardization of food products derived from this species (*17*). Therefore, this paper is aimed to investigate the contents of vitamin C, vitamin E, and carotenoids in *C. digitatum* as a suspected cause for the above-mentioned antioxidant capacity together with the possible influence of household processing conditions.

MATERIALS AND METHODS

Chemicals. All chemicals for extraction were analytical grade, and solvents for the chromatography were high-performance liquid chromatography (HPLC) quality. The carotenoid standards (*all-E*)-lutein, (*all-E*)-zeaxanthin, (*all-E*)-canthaxanthin, (*all-E*)-β-cryptoxanthin, (*all-E*)-β-carotene, (9*Z*)-β-carotene, (13*Z*)-β-carotene, and (*all-E*)-echinenone as an internal standard were purchased from CaroteNature (Lupsingen, Switzerland) and were used for identification and quantification. They were dissolved in cyclohexane/toluene (4 + 1, v/v) and stored in the dark at −30 °C. The concentration of stock solutions was calculated periodically using their absorption maxima and appropriate extinction coefficients. For preparing working solutions, stock solutions were diluted daily 1:50 with methanol (MeOH)/tetrahydrofuran (THF) (1 + 1; v/v) containing 0.1% 2, 6-di-*tert*-butyl-4-methylphenol (BHT). A mixed carotenoid standard solution was also prepared to check the peak separation. Magnesium hydroxide carbonate, sodium sulfate anhydrous, and *tert*-butyl methyl ether (TBME) were also used in carotenoids analysis.

The following reagents were used for the assessment of vitamin E: *n*-hexane/TBME (98 + 2, v/m), ethanol, TBME, petroleum ether, and internal standard: α-tocopherol acetate (Sigma-Aldrich, Taufkirchen, Germany), α-, β-, γ-, and δ-tocopherols (Calbiochem, Darmstadt, Germany), and α-, β-, γ-, and δ-tocotrienols (Davos Life Science pte Ltd., Singapore). Tocopherol and tocotrienol stock solutions in ethanol containing approximately 1 mg/mL were diluted daily 1:100−1:5000 with *n*-hexane/TBME (98 + 2, v/m) to prepare the working solutions; the internal standard was diluted 1:100. The working solutions of tocopherols and tocotrienols were used for identification and quantification. A mixture of different forms of tocopherols and tocotrienols was also prepared to check the peak separation.

DNP reagent (a mix of 2,4-dinitrophenylhydrazine, thiocarbamide, and cupric sulfate), HPLC water, metaphosphoric acid, sulfuric acid, and trichloroacetic acid were used for vitamin C assessment. From 1 mg/mL ascorbic acid in trichloroacetic acid stock solution, the calibration

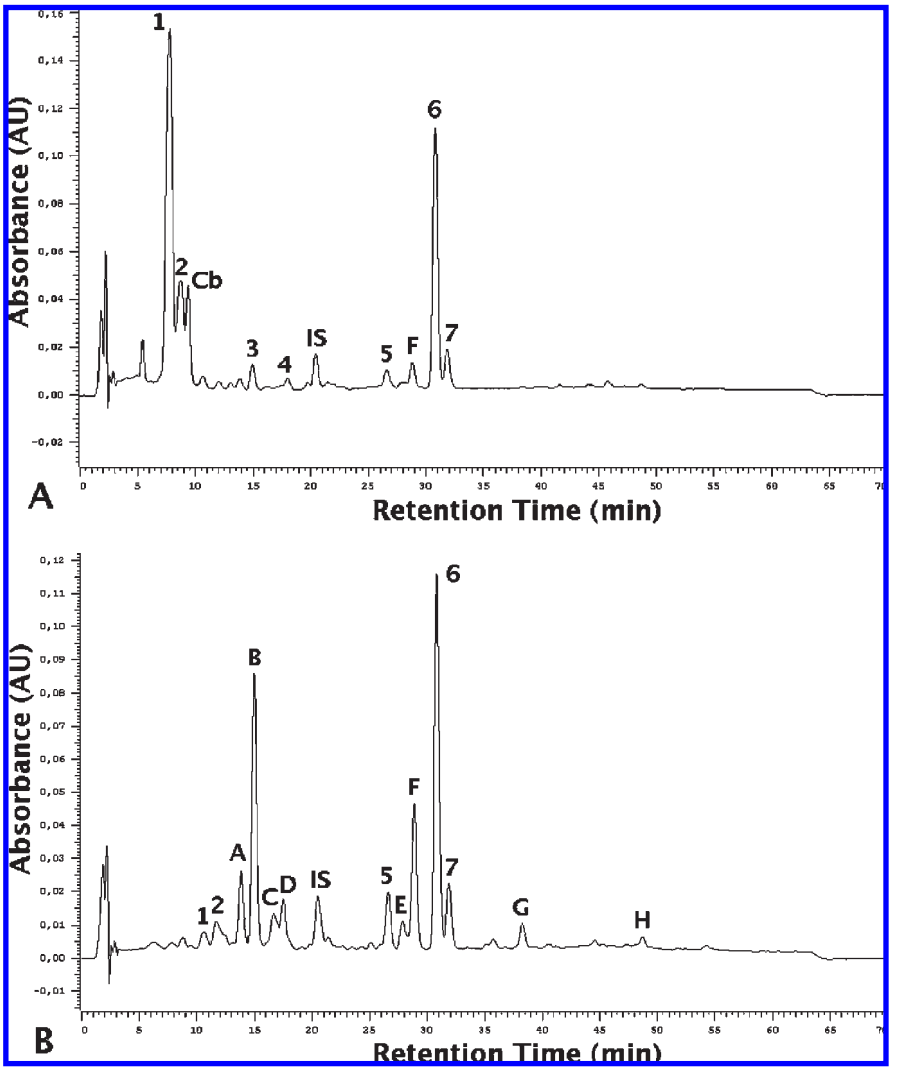

Figure 2. Chromatograms of the carotenoid extracts from *C. digitatum*: (**A**) raw material and (**B**) processed sample. IS and Cb denote internal standard and chlorophyll b, and A−H denote unknown peaks, which are still under investigation. Chromatographic conditions are described in the Materials and Methods, and peak identification (1−7) and characterization are given in **Table 1**.

solutions were done by diluting certain quantities of the stock solution in metaphosphoric acid.

Sample Preparation. Fresh leaves of *C. digitatum* were harvested from nature in August 2006 in the Baddan countryside in the southwestern highlands in central Yemen. This area was selected for sample gathering because it is away from any substantial human impact. A voucher sample was given to the Agricultural Research Centre in Taiz, Yemen. Part of the clean leaves was boiled for 30 min under pressure; then, the water was removed, and the leaf mass was mixed with a wooden spoon. The thick homogeneous stature baste was thinned into disks (8−12 cm in diameter) and dried in the sun in clean plates covered with tiny mesh, turning them upside down each day with 30 °C average temperature until complete dryness; this was called the "processed" sample. The other parts of the freshly cut leaves were dried in an oven at 40 °C for 90 min, and this sample was called "raw material". Both processed sample and raw material were used for the tests as dry materials. Both samples were packed in airtight dark containers and stored at ambient temperature (< 30 °C) for 3−5 months before use. Directly before preparation for estimation, both raw and processed samples were milled with a cyclotec mill (UDY Corp., Fort Collins, CO) (1 mm mesh).

Analysis of Vitamin C. According to the method of Roe and Oesterling (*20*), 1 g of each sample was dissolved in 5 mL of 4.5% *meta*-phosphoric acid, and the samples were shaken for 1 min and centrifuged (5000 rpm, 5 min). The aqueous phase was removed to a 20 mL flask. The extraction process was repeated twice, and the volume was completed to 20 mL by *meta*-phosphoric acid. Then, 1 mL of each sample was taken to an Eppendorf tube and centrifuged (12000 rpm, 5 min). Two hundred microliters from the supernatant of each sample, standard, and blank was taken, and 300 μL of trichloroacetic acid (5 g/100 mL) was added, mixed, and centrifuged again (12000 rpm, 5 min). Then, 300 μL of supernatant was taken, and 100 μL of DNP reagent was added. After they were shaken, all tubes were incubated in the thermal mixer at 60 ± 1 °C (800 rpm, 60 min). The tubes were removed to an ice bath for 5 min, 400 μL of sulfuric acid was added with shaking, and the tubes were left for 20 min in the dark before determining the absorbance at 520 nm. All analyses were done in triplicate.

Analysis of Vitamin E. An amount of 0.1 g of the sample was weighed into a centrifuge tube. Successively, 1 mL of distilled water, 40 μL of α-tocopherol acetat as an internal standard, 1 mL of ethanol, 1 mL of TBME, and 1 mL of petroleum ether were added. After each addition, the tubes were shaken for 30 s. Then, the samples were centrifuged (5000 rpm, 5 min), and the upper layer was transferred into a 50 mL pear-shaped flask. The extraction with 1 mL of TBME and 1 mL of petroleum ether was repeated until the solvent was colorless. The combined extracts were dried under reduced pressure at 30 ± 1 °C. The residue was dissolved in 2 mL of *n*-hexane/TBME (98 + 2, v/m) using an ultrasonic bath. Then, samples were centrifuged (14000 rpm, 5 min). The resulting solution was analyzed for vitamin E by using normal phase HPLC at 35 ± 1 °C with a Knauer Eurospher 100 DIOL-column (250 mm × 4.0 mm, 7 μm) and an appropriate guard column (5 mm × 4.0 mm, 7 μm) (Knauer, Berlin, Germany), with 1.5 mL/min of *n*-hexane/TBME (98 + 2, v/m) (**Figure 3**)

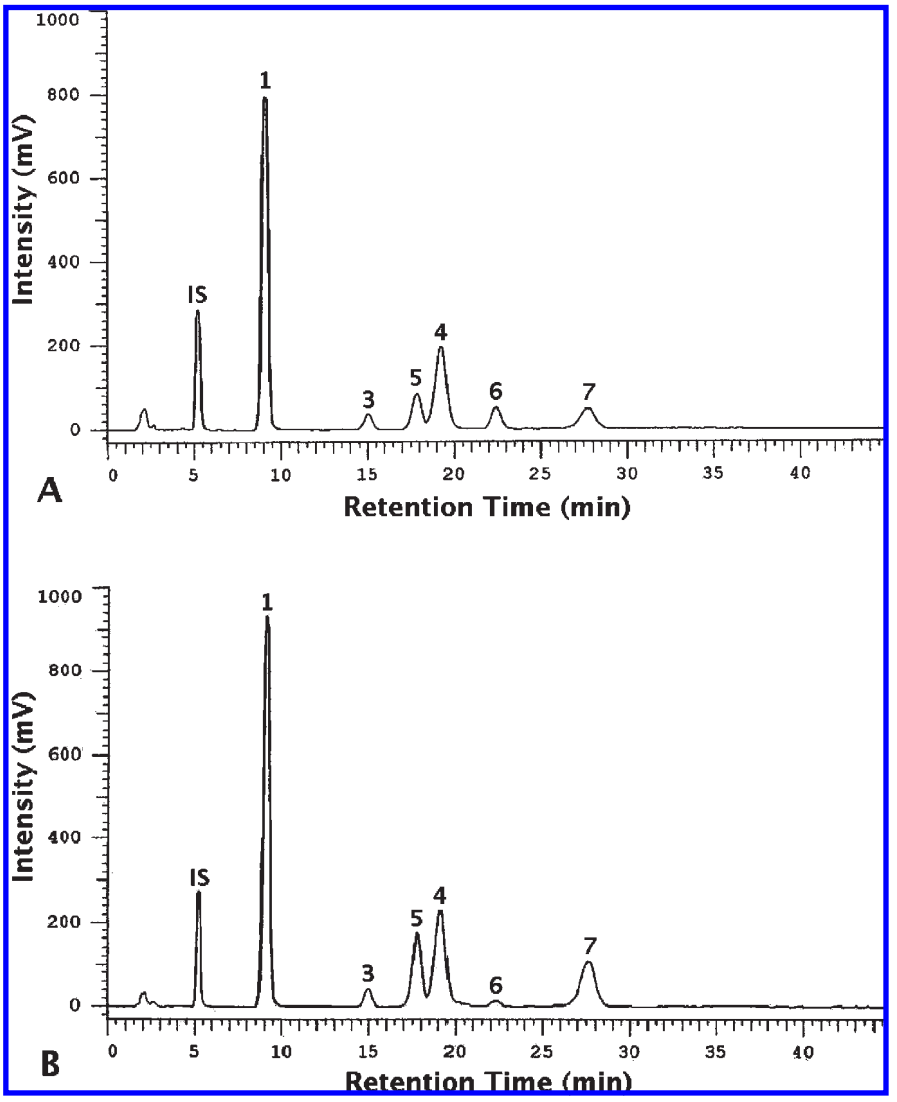

Figure 3. Chromatograms of vitamin E from *C. digitatum*: (**A**) raw material and (**B**) processed sample. Conditions are described in the Materials and Methods, and peak identification and characterization are given in **Table 2**.

with fluorescence detection (excitation, 292 nm; emission, 330 nm) (*19*). All analyses were done in triplicate.

Analysis of Carotenoids. The contents of carotenoids were analyzed by using C30-HPLC with diode array detection according to the method of Böhm (*18*). One milliliter of HPLC water was added to 1 g of the sample and mixed. After 5 min, 200 mg of magnesium hydroxide carbonate and sodium sulfate anhydrous, 200 μL of echinenone (i.e., internal standard), and 35 mL of MeOH/THF (1 + 1; v/v) containing 0.1% BHT were added. The mixture was homogenized on ice for 5 min using an Ultra Turrax (type T25, IKA-Werke, Staufen, Germany). After the residue was deposited, the solution was filtered through 390 paper (Filtrak, Niederschlag, Germany) on a Büchner funnel. The extraction was repeated at least three times until the solvent was colorless. The combined extracts were dried under reduced pressure at 30 ± 1 °C in a rotary evaporator.

The residue was redissolved in 10 mL of MeOH/THF (1 + 1, v/v) containing 0.1% BHT by using an ultrasonic bath. One milliliter of the solution was centrifuged (14000 rpm, 5 min) and then used for HPLC analysis, which was done with C30-HPLC column (250 mm × 4.6 mm, 5 μm) (Trentec, Gerlingen, Germany) and C30 guard column (10 mm × 4.6 mm, 5 μm) (Trentec) and 1.3 mL/min TBME/MeOH (gradient procedure) as the mobile phase at 17 ± 1 °C with diode array detection at 470 nm (**Figure 2**). All extractions were carried out under subdued light and were done three times for each sample.

Calculation of Vitamin A Activity. All calculations were performed on the dry mass of the raw material and the processed product; for details, see ref (*17*). Retention of each form of carotenoids, tocopherols, and tocotrienols after processing was calculated in % of that form in the raw material. The total carotenoids content was collected as mol/100 g for each form and converted to mg/100 g β-carotene equivalents for comparison purposes; also, total vitamin E was converted to mg/100 g α-tocopherol equivalents, since these two were the most abundant forms in both raw material and the processed sample (**Tables 1** and **2**). The vitamin A activities of the samples were calculated as mg retinol equivalents (RE) according to the FAO procedure (*21*). The activities in mg RE/100 g were calculated as one-sixth of β-carotene and 1/12 of other carotenoids with provitamin A activity

$$\text{mg RE/100 g} = \frac{\beta - \text{carotene}}{6} + \frac{\beta - \text{cryptoxanthin}}{12}$$

Statistical Analysis. Determinations were conducted in triplicate. Results were represented as means ± standard deviations (SDs). To ascertain differences between means, independent two sample *t* tests were done for each substance using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Methodological Considerations. Saponification was done in the beginning, but no improvement was found, which means that the samples contained no xanthophyll esters. This was anticipated since the searched material is GLV. Besides, some of the peaks were negatively affected, and small peaks became completely

Table 1. Carotenoids Composition and Vitamin A Value of Samples from *C. digitatum* before and after Processing[a]

		mg/100 g		
peak no.[b]	carotenoids	raw material[c]	processed sample[c]	retention in %
1	(*all-E*)-lutein*[f]	18.89 ± 0.73	0.19 ± 0.03	1.01
2	(*all-E*)-zeaxanthin*	9.46 ± 0.30	0.49 ± 0.01	5.18
3	(*all-E*)-canthaxanthin*	0.21 ± 0.01	ND	
4	(*all-E*)-β-cryptoxanthin*[d]	0.67 ± 0.03	ND	
5	(13*Z*)-β-carotene*[d]	1.07 ± 0.05	2.16 ± 0.18	201.87
6	(*all-E*)-β-carotene*[d]	11.38 ± 0.34	9.98 ± 0.31	87.70
7	(9*Z*)-β-carotene[d]	2.14 ± 0.07	2.24 ± 0.07	104.67
	total β-carotene[d]	14.60 ± 0.46	14.38 ± 0.46	98.56
	total carotenoids*[e]	42.20 ± 1.47	15.02 ± 0.60	35.60
	provitamin A (RE/100 g)	2.49	2.40	96.35

[a] The % of retention and provitamin A were calculated. ND, not detected. [b] Numbered according to **Figure 2**. [c] Mean ± SD ($n = 3$). [d] Denotes carotenoids with vitamin A activity. [e] Expressed as mg/100 g β-carotene equivalents; calculated from the sum of total carotenoids (mol/100 g). [f] Asterisks denote significant differences between raw and processed material ($P < 0.05$).

Table 2. Vitamin E and Vitamin C Content (mg/100 g) of *C. digitatum* before and after Processing; [a]

		mg/100 g		
peak no.[b]	vitamin	raw material[c]	processed sample[c]	retention in %
1	α-tocopherol*[e]	41.58 ± 0.41	49.57 ± 0.49	119.22
2	α-tocotrienol	ND	ND	
3	β-tocopherol*	3.00 ± 0.01	3.27 ± 0.06	109.03
4	β-tocotrienol*	22.93 ± 0.03	24.37 ± 0.37	106.31
5	γ-tocopherol*	6.16 ± 0.06	12.33 ± 0.18	200.23
6	γ-tocotrienol*	2.90 ± 0.02	0.68 ± 0.03	23.42
7	δ-tocopherol*	4.31 ± 0.11	8.36 ± 0.21	200.29
8	δ-tocotrienol	ND	ND	
	total vitamin E*[d]	82.74 ± 0.63	101.20 ± 1.38	122.31
	vitamin C[e]	49.50 ± 0.01	20.30 ± 0.02	41.01

[a] The % of retention was calculated. ND, not detected. [b] Numbered according to **Figure 3**. [c] Mean ± SD ($n = 3$). [d] Expressed as mg/100 g α-tocopherol equivalents; calculated from the sum of total vitamin E (mol/100 g). [e] Asterisks denote significant differences between raw and processed material ($P < 0.05$).

undetectable; hence, we decided to run the test without saponification. This is in accordance with previous research that emphasized that saponification can result in the destruction and/or structural transformation of some carotenoids (*22*). Therefore, HPLC methods that separate the various classes of carotenoids without saponification can be highly advantageous and provide valuable information on the identity and the levels of these compounds in their natural state in foods (*22*). All identified peaks (**Figures 2** and **3**) were determined by using standard reference materials.

In most cases, there is insufficient information on the history of the samples (i.e., cultivar, growing season, and location), the length of cooking, and the method of preparation of cooked foods (i.e., frying, steaming, boiling, baking, and microwave cooking) (*22*). Heat treatment was used to reduce the content of antinutritional substances and to diminish their effects (*23*). It is often difficult to compare analytical data about effects of cooking and processing on carotenoids levels in fruits and vegetables that have been reported in different studies. So, to determine changes in the phytonutrients content in *C. digitatum*, samples were analyzed in raw and cooked form. Estimations for *C. digitatum* are expressed in mg/100 g of dry mass, while for the rest (**Table 3**), values are shown in mg/100 g fresh matter. It is not

Table 3. Vitamin C, Vitamin E and Lutein Content of the Raw Material (rM) and the Processed Sample (pS) of *C. digitatum* as Compared with Other Well-Known Sources from German Food Composition Table (*24*), Quantified in mg/100 g

	vitamin C	vitamin E	lutein
C. digitatum (rM)[a]	49.50	82.74	18.9
C. digitatum (pS)[a]	20.30	101.20	0.19
asparagus	20.00	2.00	
blackberry	17.00	0.72	
blueberry	20.00	2.10	
broccoli	100.00	0.62	23.20
cabbage	50.00	1.70	
carrot	7.00	0.46	3.00
cauliflower	65.00	0.09	
corn salad	35.00	0.60	
garlic	14.00	0.01	
gooseberry	35.00	0.72	
grape	4.00	0.66	
green pepper	120.00	2.50	
kale	50.00	2.50	
leeks	25.00	0.53	
lettuce	13.00	0.60	11.30
onion	7.00	0.08	
pumpkin	12.00	1.10	9.20
raspberry	25.00	0.91	
red cabbage	55.00	1.70	
rhubarb	10.00	0.25	
rose hip	1250.00	4.20	
spinach	50.00	1.40	69.50
strawberry	65.00	0.12	
tomato	19.00	0.82	1.20

[a] For *C. digitatum*, estimations are expressed in mg/100 g of dry mass, while for all other substances, they are given in mg/100 g fresh matter (for an explanation, see the text).

meaningful to give results for *C. digitatum* per 100 g fresh matter, as it is used after redissolving in water in different concentrations according to the taste needs and person involved.

Vitamin C Content. The plant *C. digitatum* is a very promising and inexpensive source of vitamin C as raw material; hence, it could be used commercially for the extraction of ascorbic acid for culinary and pharmacological applications. We found 49.5 mg/100 g in the raw material and 20.3 mg/100 g in the processed material; this concentration could be compared to that in carrot, cauliflower, broccoli, corn salad, lettuce, leeks, Brussels sprouts, red cabbage, cabbage, rhubarb, asparagus, spinach, kale, onion, garlic, bumpkins, tomato, blackberry, strawberry, blueberry, raspberry, gooseberry, grape, kiwi, orange, lemon, and bananas (see **Table 3**) (*24*).

The vitamin C content of *C. digitatum* was decreased by the household processing; only 41% was retained after processing. However, this is rather high as compared to only 15% retention in all analyzed hot and sweet pepper cultivars, which are one of the important sources of vitamin C, in which the content in fresh fruits ranged from 101.2 to 167.5 mg/100 g of fresh weight (*13*). Because vitamin C is water-soluble, presumably reasonable amounts were discarded with the water during the processing of *C. digitatum* (see Sample Preparation).

Vitamin E Content. Many forms of vitamin E were found in both sample types of *C. digitatum*, but amounts were higher in the processed sample with remarkably very high extractability of especially γ-tocopherol and δ-tocopherol; only γ-tocotrienol was negatively affected by processing. In total, vitamin E was even enhanced by 22% through household processing (cf. **Table 2**). This high content of vitamin E in the processed sample could be attributed to two reasons: The processing may cause denaturation of proteins and a complete destruction of cell walls and cell

organelles and consequently result in liberation of vitamin E from the lipids, which then becomes more available for extraction. The other reason is that relatively thick and very dry discs were produced (as processed sample), which kept vitamin E and other functional food ingredients away from contact with oxygen and light, therefore, remaining intact, as compared with the thin pieces of dry leaves (as raw material). This is in accordance with the well-known stability of some forms of vitamin E like α-tocopherol, which significantly contribute to the stability of olive oil during potato frying (*25*).

C. digitatum proved to be an outstanding, very wealthy source of vitamin E: It contained 82.7 mg/100 g and 101.2 mg/100 g in the raw and the processed material, respectively; this concentration could be compared to green pepper, kale, blueberry, asparagus, cabbage, spinach, and many other fruits and vegetables (cf. **Table 3**) (*24*). Moreover, the vitamin E content of *C. digitatum* in both forms as a rich source of vitamin E could be compared with the well-known sources rich in vitamin E, as butter contains 2 mg/100 g, medium-fat margarine contains 6 mg/100 g, cocoa butter contains 1.1 mg/100 g, palm oil contains 9.5 mg/100 g, sesame oil contains 3.5 mg/100 g, and walnut oil contains 20.0 mg/100 g (*24*). Because the main sources of vitamin E are animal fat and plant oils, such extraordinary vitamin E-rich green leafy vegetables are important in food supplementation to avoid the high consumption of fats and oils. The high vitamin E dose of *C. digitatum* could be especially important as a food supplement for people with fats and oils malabsorption since they may suffer from vitamin E deficiency. Moreover, *C. digitatum* could be a proper resource for the extraction of pure vitamin E for medicinal applications and cosmetics and food industries.

C. digitatum also had a highly remarkable content of certain forms of vitamin E (cf. **Table 2**). For example, α-tocopherol reached 49.6 mg/100 g in the consumed form, which is rather high as compared to 61 mg/100 g in sunflower, recognized as one of the richest sources of this form and to 18 mg/100 g in canola oil and 12 mg/100 g in olive oil, respectively. According to the German Food Composition Table (*24*), *C. digitatum* seems to be the richest source in β- and γ-tocotrienol, which is very rare in nature as compared with the tocopherol counterpart class.

Carotenoids Content. The results showed that there is a high level of total carotenoids in *C. digitatum*: About 42 mg/100 g was determined in the raw material (dry sample), and 15 mg/100 g was still found in the processed form (dry sample) (see **Table 1**). These results are remarkable when compared to contents in vegetables, like 2.1 mg/100 g in beans, 8.2 mg/100 g in broccoli, 12.2 mg/100 g in chive, 2.2 mg/100 g in green bell pepper, 6.2 mg/100 g in lettuce (curly), 11.5 mg/100 g in lettuce, 23.2 mg/100 g in parsley, 3.2 mg/100 g in peas, and 15.6 mg/100 g in spinach, all as fresh samples (*26*). Our result is also in accordance with a survey study on selected leafy vegetables with medicinal value, but less commonly used for nutritional purpose, which generally contains higher levels of lutein than β-carotene (*27*). Moreover, the total carotenoids and provitamin A contents of *C. digitatum* were higher than those in *Brassica oleracea*, *Hydrocotyle asiatica*, and *Mentha spicata*, where total carotenoids were 10.32, 26.49, and 33.21 mg/100 g, respectively (*27*). *C. digitatum* also contained more provitamin A than *Allmania nodiflora*, *Beta vulgaris*, *Cucurbita maxima*, and *Murraya koenigii* (*27*).

Different classes of carotenoids differ in their stability toward heat treatment (*22*). The stability of carotenoids is also different among foods if the same processing and storage conditions are used. Thus, optimum conditions for carotenoids retention during preparation and processing differ from one food to another (*28*). Lutein and zeaxanthin are xanthophyll carotenoids found in a wide variety of plant foods, especially in GLVs. As in other GLVs, lutein is the most common vitamin A inactive carotenoid in *C. digitatum*. About 18.9 mg/100 g was determined in the raw material and 0.19 mg/100 g in the processed material. A very poor retention after processing of only 1% was observed. However, peaks A and B in the processed sample could be a derivative of lutein (the structural elucidation of these peaks is still in progress and will be reported separately) (**Figure 2**). Anyway, the lutein content in the raw material of *C. digitatum* can be compared to the lutein contents in carrot, broccoli, pumpkin, spinach, lettuce, and tomato, which contained 0.3, 2.3, 1.1, 6.9, 0.9, and 0.12 mg/100 g, respectively, expressed per 100 g edible portion (*24*).

Canthaxanthin and zeaxanthin are very rarely reported in GLVs, but zeaxanthin was found in both the processed sample and the raw material with less extent than lutein, while zeaxanthin had a better retention after processing than lutein. Lutein, zeaxanthin, canthaxanthin, and β-cryptoxanthin are very labile toward heat treatment and suffer tremendous losses (**Figure 2**). Only 1.0, 5.2, and 0%, respectively, were retained in the processed sample. Thus, to increase the dietary intake of carotenoids, including enhancement of bioavailability, it is recommended in general strategies to optimize cooking and processing conditions such that appreciable losses of carotenoids are prevented while the bioavailability is increased (*27*).

The high content of vitamin C in the matrix, which lowers the pH, together with high temperature and pressure during processing, resulted in a dramatic loss of lutein. This is in accordance with previous findings where the level of lutein in the extract of the autoclaved sample of spinach was substantially reduced, while in other green vegetables that contained weak organic acids, such as oxalic acid, lutein did not undergo dehydration, which finally led to degradation under these conditions (*22*).

The vitamin A active carotenoid β-carotene was unlike lutein under the household cooking conditions (see Sample Preparation); all isomers of β-carotene are quite heat resistant. β-Carotene levels in the processed sample as compared with raw material were not statistically different; 98.6% of β-carotene was retained in the processed sample (see **Table 1**). This high retention could be attributed to the inactivation of oxidative enzymes by the heat treatment, which prevents further and greater losses during storage (*27*, *29*). Moreover, the (13*Z*)-β-carotene isomer was significantly higher after processing, while (*all-E*)-β-carotene decreased in the same level, which implies that there is conversion of (*all-E*)-β-carotene to the (13*Z*)- and (9*Z*)-isomers (**Table 1**). This is obviously attributed mainly to the processing conditions but might be also a result of the UV light during the subsequent sun drying. The rise of β-carotene isomers was also reported in previous research, among them a research on green beans, broccoli, and spinach cooked under various conditions (*22*).

Provitamin A Content. It is well-known that VAD is a problem in less-developed areas of the world. Dark green leafy vegetables are the most common rich sources of provitamin A. Relatively easy to produce and available practically all year round, they are inexpensive and accessible sources of provitamin A for people in most of the developing world (*29*). In GLVs, β-carotene is essentially the sole contributor to vitamin A activity, while α-carotene and α- or β-cryptoxanthin are being reported only occasionally and at very low levels. However, the β-carotene content of leafy vegetables can vary markedly (*29*). Despite the importance of the provitamin A carotenoids in limiting eye illnesses, other carotenoids and vitamin E also play crucial role in that regard. In prospective observational data from large populations of female health professionals, a higher intake of the carotenoids lutein and zeaxanthin could reduce the risk of developing cataracts by about 18%, while a high intake of

vitamin E from food and supplements was associated with a 14% lower risk of cataract (*30*). Food based on *C. digitatum* appears to be a promising source in terms of provitamin A and also other phytonutrients like lutein, zeaxanthin, and vitamin E that sustain healthy eyes. The provitamin A content was found to be 2.49 and 2.40 mg RE/100 g in the raw material and the processed sample, respectively, corresponding to 96.4% retention after processing.

According to the central statistical organization in Yemen, there are 76000 blind people in Yemen, that is, 35 blind for each 10000 of the population, most of them living in rural areas (*31*). Another study was done in 18 districts of western Yemen on 338 children ages 1−5 years aimed to link the prevalence of xerophthalmia and night blindness with the extent of VAD (*15*). They found that about 2.2% of the children had active xerophthalmia; children ages 4−5 were more likely to have xerophthalmia than those less than 4 years, and boys were more likely ill than girls. Of the xerophthalmia cases, 77.8% had Bitot's spots, and 71% had spots in both eyes. The prevalence of Bitot's spots exceeded the minimum criteria for public health significance of xerophthalmia (1.72 vs 0.50%). The prevalence of night blindness stood at 0.45%. Children with xerophthalmia had much lower retinol levels than those without xerophthalmia (11.4 vs 18.8 μg/dL; $P < 0.001$). Likewise, children with night blindness had lower retinol levels than those without night blindness (10.9 vs 18.3 μg/dL; $P < 0.001$). The results of this study were very alarming; it came out with the conclusion that xerophthalmia and VAD are public health problems in western Yemen (*15*).

Anticipated Synergistic Effect. The beneficial effects of vitamin A, β-carotene, and lutein or zeaxanthin supplementation on human visual performance are well-known (*32*). Thus, data on carotenoid and provitamin A content of such a free and reasonably accessible source as *C. digitatum* may provide information to consumers and public health workers to support the dietary daily carotenoids intake in general and be helpful to create nutritional awareness among various target vulnerable age groups and to assess their relationship to health and disease. Other researches showed that both Alzheimer's and multi-infarct dementia patients had significantly lower levels of vitamin E and β-carotene than controls (*33*). Recently, use of vitamin E and vitamin C supplements in combination for Alzheimer's disease in elderly population was found to be associated with reduced prevalence by 78% and incidence by 64% (*34*).

With these high concentrations of carotenoids and vitamins, synergistic action is highly anticipated in vivo after a meal containing *C. digitatum*. This conclusion is strongly supported by previous findings, where the dose-dependent first evidence of a prooxidant in vitro effect of β-carotene under 100% oxygen pressure in a biological membrane model was illustrated, and the existence of cooperative interactions between β-carotene and α-tocopherol was shown (*35*). Meanwhile, it was pointed out that α-tocopherol should always function as an antioxidant, as long as the concentration of other coantioxidants, such as vitamin C, is high enough to convert α-tocopheroxyl radical back to α-tocopherol. Considering vitamin C's high concentration in human plasma, it is highly probable, in vivo, that vitamin E should act as an antioxidant regardless of the oxidative conditions (*36*).

Future Enhancement of the Product. *C. digitatum,* both raw material and processed sample, contained reasonably higher total phenolics contents and higher antioxidant capacities than asparagus and broccoli (rich sources for theses phytonutrients), although *C. digitatum* antioxidant capacities did not correlate with the total phenolics content (*17*). This antioxidant capacity was measured in hydrophilic matrix, resulting mainly from vitamin C and phenolic compounds. The high content of vitamin E and also the remarkable contents of carotenoids lead to the expectation that the antioxidant capacity of *C. digitatum* in the hydrophobic matrix might be even important.

Although traditional sun drying is the cheapest and most accessible means of food preservation, it is well-known that it causes considerable carotenoid destruction (*27*). So, drying in simple inexpensive solar dryers is highly recommended and can appreciably reduce losses in carotenoids. Modifications of the traditional cooking practices should be tried first (*29*). Various means of cooking (microwaving, boiling, and steaming) must be studied extensively to see the effect on the qualitative and quantitative distribution of carotenoids in *C. digitatum*. Optimization of processing time and temperature and of storage conditions is also recommended (*29*). Exclusion of oxygen may be assured by suitable packaging methods (e.g., through vacuum or hot filling, oxygen-impermeable packaging, or inert atmosphere). Low temperatures and protection from light can diminish carotenoid decomposition during storage, keeping storage time at a minimum (*27*).

The harvesting time of *C. digitatum* should also be studied to gain the best yield of this functional food ingredient. For example, in *B. oleracea*, carotenoid concentrations were significantly higher in the second year than in the first year (*37*). In lettuce and endive, the β-carotene content of the mature leaves was found to be three times greater than that of the young leaves taken from the same bunches of vegetables (*38*).

Many enzymes and secondary compounds of higher plants have been used in in vitro experiments to show protection against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (*7*). In that regard, some active phytochemicals were quantified, among them luteolin, in which bioactivity was tested in vitro (to be reported separately). More functional food ingredients and non-nutrient components must be determined in the fresh material to see the effect of drying and storage. It has become evident that several research questions need to be addressed soon; the high carotenoids contents suggest to progress in flavor and aroma constituents determination since many aroma compounds are generated from carotenoids by food cooking and processing (*3*); one of the main causes for using the processed sample is for its aroma.

Animal model experiments should be established soon with suitable strains vulnerable to vascular disorders and cancer. Serum and plasma response assessment will also be useful to study the bioavailability of these phytochemical micrometerutrients from *C. digitatum* after consumption, and the dosages that could promote health among the consuming public should be determined. Our findings proved that *C. digitatum* may become a new food source, being very rich in vitamin E, vitamin C, provitamin A, and other important carotenoids. Meanwhile, through this work on *C. digitatum*, we established research on Vitacea family as an important GLVs candidate for functional food ingredients.

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